Binding and cytotoxicity of cerato-ulmin to elm cells.

BINDING AND CYTOTOXICITY OF CERATO-ULMIN TO ELM CELLS

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SUMMARY

In vitro cultivated elm cells were used to study the cytotoxicity of cerato-ulmin (= CU), a hydrophobin produced by *Ophiostoma ulmi* and *O. novo-ulmi*, two causal agents of Dutch elm disease (= DED). Purified CU was found to bind to elm cells and cause a dose-dependent death both on cells derived from a DED susceptible (C06) and a moderately resistant (196/6) elm clone. Indirect immunofluorescence (IF) tests, using a rabbit anti-CU antiserum, indicated that homogeneous CU binds to elm but not to cells of fennel and pear, two non-host species of *Ophiostoma*. The binding was demonstrated by quantitative ELISA on extracts from elm cells incubated with purified CU and by measuring the decrease in CU concentration in the incubation mixture. The equation of the saturation binding curve indicated that the maximum quantity of bound CU for elm clones C06 and 196/6 were similar (351.92 and 355.59 pmoles mg^{-1} d. wt elm cells, respectively), as similar were the equilibrium dissociation constants (7.09 and 8.08 µM CU, respectively). The increase in bound CU correlated with the intensity of the fluorescence signal on elm cells and a higher mortality. Elm cell-*Ophiostoma* spp. dual cultures in Takai or Murashige & Skoog-derived media were used to test the ability of elm cells to support growth of *Ophiostoma* fungi and CU production. Cells derived from two elm clones supported both abundant fungal growth and CU production. Fungal growth was supported by cells of the *Ophiostoma* non-hosts fennel and pear, whereas CU was only produced in the presence of fennel cells. The binding and direct cytotoxicity of CU on elm cells support its role as a pathogenicity factor in DED.

Key words: *Ophiostoma* spp., *Ulmus* spp., toxin binding, *in vitro* fungus-plant cells dual cultures.

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INTRODUCTION

The Ascomycetes *Ophiostoma ulmi* (Buisman) Nannfeldt and *O. novo-ulmi* Brasier have caused two successive pandemics of a very destructive disease of numerous plant species belonging to genus *Ulmus*, which is known as Dutch elm disease (DED). The second pandemic of DED, caused by *O. novo-ulmi* with the subspecies *novo-ulmi* and *americana* (Brasier and Kirk, 2001), formerly designated Eurasian (=EAN) and North-American (=NAN) races, respectively, was more severe than the first one and almost destroyed the European and North-American native elms.

The phytotoxic hydrophobin cerato-ulmin (CU) (Bowden *et al*., 1994; Scala *et al*., 1994; Stringer and Timberlake, 1993; Yaguchi *et al*., 1993) is a component of the mycelial surface of *O. novo-ulmi*, *O. ulmi* and *Ophiostoma quercus* (Georgevitch), the latter reported as *Ophiostoma piceae* (Munch) H. and P. Sydow (Scala *et al*., 1997a; Svircev *et al*., 1988; Takai and Hiratsuka, 1980), and is released in shake liquid culture by only *O. ulmi*, *O. novo-ulmi* and *Ophiostoma himal-ulmi* (Brasier, 1991; Brasier *et al*., 1990; Brasier and Mehrotra, 1995; Nordin *et al*., 1987; Richards and Takai, 1973; Scala *et al*., 1997a). The role of CU in DED pathogenesis is still being debated (Del Sorbo *et al*., 2002). Many evidences suggest a key role of the toxin in virulence of DED pathogens. For instance, in DED-susceptible elm genotypes, homogeneous CU causes internal and external symptoms identical to those of DED (Takai, 1974; Takai *et al*., 1983; Takai and Hiratsuka, 1984), alteration of several physiological parameters of both cuttings and leaves (Richards, 1993; Richards and Takai, 1984) and inhibition of division of *in vitro* deep-plated cells (Tegli *et al*., 1993).

However, there is evidence against a role of CU in virulence. Some naturally-occurring (Brasier *et al*., 1995) or laboratory-induced (Tegli and Scala, 1996) mutants of *O. novo-ulmi*, which lost the ability to produce CU *in vitro*, maintained their virulence on elm. Transformation-mediated disruption of the CU-encoding gene in *O. novo-ulmi* did not determine changes in virulence on rooted cuttings and seedlings of highly susceptible elm genotypes (Bowden *et al*., 1996). Transgenic expression of the *cu* gene of *O. novo-ulmi* in *O. ulmi*, a less virulent species, determined an increase in conidial

adhesion properties to DED vectors and resistance to dessiccation but not an increase in virulence on *U. americana* plants (Temple *et al*., 1997). Et Touil *et al.* (1999) reported that virulence of two strains of *O. novo-ulmi*, which produce similar amounts of CU *in vitro*, depends on the single nuclear gene *Pat1*.

CU production by DED pathogens in axenic culture depends on several factors, including fungal genotype. Isolates belonging to both *novo-ulmi* and *americana* subspecies of *O. novo-ulmi* produce more CU than *O. ulmi*, often in amounts significantly correlated to their respective pathogenicity levels on elm (Brasier, 1991; Brasier *et al*., 1990). Temperature affects CU production, as the two species have two different temperature optima: 21-23°C for *O. novo-ulmi* and 31-33°C for *O. ulmi* (Tegli *et al*., 1994). CU production is also influenced by the carbon and nitrogen source, as well as by salt concentration of culture medium (Takai, 1978).

The role in pathogenesis of toxins produced *in vitro* by phytopathogenic fungi has widely been discussed. Mitchell (1981) assumed that toxins produced *in vitro* by phytopathogenic fungi are also produced *in planta* during pathogenesis. On the other hand, Gilchrist (1983) suggested that phytotoxic metabolites present in fungal culture filtrates could be culture-derived artifacts, normally not produced during pathogenesis. Yoder (1980; 1981) stated that virulence cannot be always correlated with the quantity of toxin produced in culture. CU was detected in both *O. novo-ulmi* naturally-infected and artificially-inoculated elms (Richards and Takai, 1988). Scala *et al.* (1997b) reported that the amounts of CU produced *in planta* are correlated with those produced in vitro by *O. novo-ulmi* and *O. ulmi*. Moreover DED symptoms were associated with high CU contents on leaves. Transgenic expression of the *cu* gene originating from a high CU-producer isolate of *O. novo-ulmi* in *O. quercus*, a saprophyte on elm, enables the fungus to cause typical DED symptoms (Del Sorbo *et al*., 2000).

In this paper, we studied the effect of the addition of CU to elm cell cultures. This system simulates *in vitro* the condition occurring *in planta* during pathogenesis. The finding that CU binds and determines toxicity to elm cells contributes to a better under understanding of the role of CU in DED. We also analysed *in vitro Ophiostoma* spp. - elm cell dual cultures with the aim to elucidate whether CU production by the DED fungi is influenced by the presence of host plant cells.

MATERIALS AND METHODS

Fungal cultures. The isolates H328 and H327 of *O. novo-ulmi* ssp. *novo-ulmi*, the isolates 182 and H351 of *O. novo-ulmi* ssp. *americana*, and the isolates E2, R21 and Yu99 of *O. ulmi* are described in previous papers (Scala *et al*., 1994; Tegli *et al*., 1994; Tegli and Scala, 1996). The isolate Yu16 of *O. ulmi* was provided by Prof. L. Mittempergher (Istituto per la Protezione delle Piante, CNR, Firenze, Italy). The isolate H162 of *O. novo-ulmi* ssp. *americana* was from the collection of Prof. C. M. Brasier (Forest Research Station, Alice Holt Lodge, Farnham, UK). Long- and short-term maintenance of isolates and culture conditions are reported by Scala *et al.* (1994). Budding spores (blastoconidia) were obtained in shake culture in the standard Takai liquid medium (TK) (Takai, 1978) amended according to Scala *et al.* (1994). Blastoconidia concentration was directly estimated by a hemocytometer.

In vitro **callus cultures and cell suspensions**. *In vitro* elm callus cultures were obtained from two different elm clones, C06 (very susceptible to DED) and 196/6 (moderately resistant to DED). Callus was initiated from young leaves previously surface-sterilised under vacuum in 1% commercial sodium-hypoclorite solution for 30 min and then rinsed four times in sterile distilled water. Leaf segments, including a portion of the mid-vein, were plated on solid Murashige & Skoog medium (MS) (M0404, Sigma-Aldrich, Milan, Italy) supplemented with 2 µM 2,4 dichlorophenoxyacetic acid, 5 µM kinetin, 2 mg ml^{-1} glycine, 10 mg ml^{-1} ascorbic acid, 30 g l^{-1} sucrose and 7 g l^{-1} agar. Cultures were incubated in the dark at 23±1°C. Proliferating callus cultures were then routinely transferred to fresh solid MS every 4 weeks. A fine elm cell suspension was obtained by inoculating 10 g of friable callus in 100 ml of liquid MS medium. Agitation of the culture at 100 rpm for 2 days caused the release of single cells and small cell aggregates into the medium. The cell suspension was filtered through a 60 mesh stainless steel screen, washed twice with fresh medium and used for experiments.

Fennel and pear cell suspensions were obtained from actively proliferating callus cultures using the same procedure described for elm cells.

Cerato-ulmin - elm cell interaction. Homogeneous CU was obtained from the culture filtrate of the EAN *O. novo-ulmi* H328 (Scala *et al*., 1994). Interaction between homogeneous CU and elm cells was studied by two different systems. In the first one, different amounts of CU were added to 10 ml aliquots of a standard $5 \cdot 10^4$ ml^{-1} cell suspensions of elm clones C06 or 196/6 in order to have a CU concentration range from 0.1 to 32 µM. Elm cell viability and IF assays were performed 3 days after CU administration. Data were collected from two independent experiments, each performed in triplicate. The effect of the same range of CU concentrations was tested in 10 ml suspensions of fennel or pear cells ($5 \cdot 10^4$ ml^{-1}).

In the second one, cell suspensions of elm clone 196/6 containing increasing cell densities (ranging from $0.25 \cdot 10^4$ to $2 \cdot 10^4$ cells ml^{-1}) were incubated in presence of a fixed CU concentration (5 µM) at 23°±1°C in agitation (100 rpm). The concentration of CU in solution was measured by indirect ELISA after 1.5, 3 and 6 days

of incubation. The experiment was done in triplicate and repeated two times using different batches of cells.

Evans blue assay. Elm cell viability was evaluated using the Evans blue stain according to the method of Baker and Mock (1994), with slight modifications. One-ml aliquots of cell suspensions were transferred in test tubes and incubated with 0.5 ml of 0.1 % (w/v) aqueous Evans blue for 5 min. Cells were washed in a Sartorious funnel fitted with 3 µm Millipore membranes with distilled water until no further blue eluted from cells. After washing, cells were gently transferred from membranes in 1.5 ml tubes containing 500 µl of 1 % (w/v) sodium dodecyl sulphate (SDS) aqueous solution and 100 µg of carborundum. Cells were ground using a micro-pestle, and the homogenate diluted with 500 µl of distilled water. Tubes were then briefly vortexed, incubated for 2 h at room temperature, and centrifuged at 9,000 *g* for 3 min. A 0.8 ml aliquot of the supernatant was removed, and the absorbance determined spectrophotometrically at 600 nm (A_{600}). The freshly prepared cell suspension was assumed to contain 100% of viable cells, and gave a A_{600} of 0.300. A suspension containing 100% of dead cells, obtained by boiling cells for 10 min, gave a A_{600} of 0.700. Identical values of A_{600} were given by an elm cell suspension exposed to lethal concentrations of CU, whose inability to reproduce was checked by plating (Tegli *et al.*, 1993). The percentage of dead cells in each sample was calculated as follows:

$$\% \text{ of dead cells} = \frac{A_{600} \text{ of sample} - 0.300}{0.700 - 0.300} \cdot 100$$

Immunofluorescence (IF) assay. Cell suspensions samples (2 ml) were collected and centrifuged for 10 min at 5,000 *g* at 4°C. The pellet was washed three times with 0.9% (w/v) NaCl and once with distilled water and then resuspended to a concentration of 10^4 plant cells ml^{-1}. For IF assay, 5 µl of this suspension were dispensed onto 12-well Multitest slides (Flow Laboratories, McLean, VA, USA) and dried at 55°C. Wells were washed with PBS, filled with 20 µl of anti-CU antiserum diluted 1:100 in PBS and incubated for 1 h at room temperature. Control wells were prepared by using preimmune serum diluted 1:100. After three washings with PBS, wells were filled with 20 µl of a 1:40 dilution of goat antirabbit IgG fluorescein isothiocyanate conjugate (Sigma-Aldrich, Milan, Italy) in PBS and incubated for 1 h at room temperature. Slides were washed with distilled water and examined with a Zeiss Axioskop microscope equipped for epifluorescence with an HBO 50 lamp (exciter blue filter, BP 450-490 and barrier filter LP 520).

The intensity of fluorescence was recorded on an arbitrary 0-3 scale, where 0 = no visible fluorescence; 1 = weak fluorescence; 2 = moderate fluorescence; 3 = intense fluorescence.

Fungus-elm cell suspensions dual cultures. Fungus-elm cell dual cultures were obtained by inoculating 50 ml Erlenmeyer flasks with 10 ml of a $5 \cdot 10^4$ ml^{-1} elm cell suspension and $5 \cdot 10^5$ ml^{-1} blastoconidia. Four different liquid media were used: TK without yeast extract (YE) (TK_0), TK_0 additioned with 0.3 g l^{-1} YE (TK0.3YE), MS, MS additioned with 2 g l^{-1} YE (MS2YE). Scala (1997) demonstrated that growth of *Ophiostoma* spp. and production of CU in quantities similar to those obtained in the standard medium TK (which contains 2 g l^{-1} YE), are supported at least by 0.3 and 2 g l^{-1} YE added to TK_0 and MS, respectively. Dual cultures were incubated for 10 days in a rotary shaker at 100 rpm at 23±1°C in the dark. Dual cultures were also prepared using fennel or pear cells and grown in similar conditions.

CU assays. CU concentration was determined by two different assays. CU concentration in liquid media was determined according to the turbidimetric method of Takai and Richards (1978) as modified by Scala *et al.* (1994) and expressed as CU production index (CPI). This index is based on a linear relation between the optical density at 400 nm of a shaken sample and the decimal logarithm of the dilution factor. A CPI value of 100 corresponds to a CU concentration of 26.4 µg ml^{-1} (Scala *et al.*, 1994).

CU bound to elm cells was quantitatively determined by ELISA in cell extracts. An antiserum specific for CU from *O. novo-ulmi* isolate H328 was used (Scala *et al.*, 1994). Elm cell suspensions were collected and centrifuged at 5,000 *g* for 15 min at 4°C. The pellet was washed three times with 0.9% (w/v) NaCl and once with distilled water, and then freeze-dried. A freeze-dried aliquot (20 mg) of each cell suspension sample was extracted for 2 min with 500 µl of 60% (v/v) ethanol and centrifuged for 5 min at 13,000 *g*. The supernatant was dried under vacuum. The residue was solubilized in 500 µl phosphate buffered saline 0.15 M, pH 7.2 (PBS) and used for coating of wells of Falcon 3911 Microtest flexible plates (Becton Dickinson Labware, Oxnard, CA, USA). Test samples (50 µl) were added to triplicate wells for 3 h at 37°C. The wells were washed three times with PBS and any remaining binding sites were blocked with 50 µl/well of 0.5% (w/v) gelatine in PBS for 2 h at 37°C. Each assay included a standard calibration curve that was generated by preparing serial dilutions of homogeneous CU in PBS. Wells coated with 50 µl of extracts from cells inoculated with distilled water served as blanks. Absorbance values for the blanks were subtracted from all test values. Following saturation, 50 µl of anti-CU antiserum diluted 1:1000 in PBS containing 0.25% (w/v) gelatine and 0.2% (v/v) Tween 20 were added. Rabbit preimmune serum was used in control wells. Plates were incubated overnight at 4°C and washed three times with PBS. Fifty µl/well of goat anti rabbit IgG-peroxidase conjugate (Sigma-Aldrich, Milan, Italy) diluted 1:2000 in PBS

containing 0.25% (w/v) gelatine and 0.2% (v/v) Tween 20 were added and allowed to incubate for 2 h at 37°C. After three washes with PBS, 150 µl /well of substrate solution (0.4 mg ml^{-1} *o*-phenylenediammine dihydrochloride; 0.012% (v/v) H^2O^2 in 0.1 M citrate-phosphate buffer pH 5.0) were added. The A_{492} was measured after 5 min of incubation with a Titertek Multiskan Plus MKII plate reader (Flow Laboratories Inc., McLean, VA, USA). The concentration of CU in individual samples was determined by comparing the mean A_{492} obtained for triplicate wells to a standard curve using linear regression analysis. Standard calibration curves had a linear correlation coefficient ≥0.96 using purified CU over a concentration range of 0.1-30 µg ml^{-1}. The conditions of the assay were optimised to have an A_{492} of about 0.5 for the wells containing the highest concentration of CU. Slopes similar to that of standard curves were obtained with serial dilution curves of samples. The sensitivity of the ELISA for CU was of 100 ng ml^{-1}. Negative samples always yielded an $A_{492} \leq 0.020$.

Statistical analysis. Data of CU production by *O. ulmi* and *O. novo-ulmi* EAN and NAN were analysed by ANOVA, fixed model. Homogeneous groups were identified by means of Tukey HSD test.

RESULTS

CU binds to elm cells and determines mortality in a dose-dependent manner. Preliminary experiments were performed in order to find the optimal conditions, in terms of cell concentration and incubation time, to study the binding of CU to elm cells. In these experiments, an amount of CU (5 µM) sufficient to cause wilt of elm cuttings (Scala *et al*., 1994) was incubated with suspensions of elm cells for different periods of time. Daily measurements of unbound CU revealed a slow and gradual decrease in CU concentration paralleled by an increase in fluorescence of elm cells in IF assays. On the basis of these results we found that a three-day-incubation was suitable for studying the binding parameters of CU to elm cells and chose CU concentration range and cell density to be used for subsequent experiments.

Viability of cells was measured on a DED susceptible (C06) and a moderately resistant (196/6) elm clone in the presence of increasing concentrations of CU. For both clones a drastic decrease (about 75%) in the percentage of viable cells was observed at CU concentrations above 2 µM. At CU concentrations above 8 µM the mortality of elm cells, as calculated with the Evans blue assay, increased to over 90%. No differences in sensitivity to the cytotoxic effect of CU was found between cells of the two elm clones tested. Binding of CU to cells of both clones, as revealed with fluorescein-labeled anti CU antibodies, progressively increased with CU concentration. Cells appeared faintly fluorescent starting at 0.3 µM CU. At the higher CU concentrations tested, which determined nearly 100% mortality, the fluorescence was intense. An increasing relative fluorescence index was always associated with decreases in cell viability, indicating a dose-dependent effect of bound CU on elm cell mortality. When the effect of purified CU was tested on fennel or pear cells no fluorescence neither cytotoxicity were observed. These observation give a further indication that CU binding is specific for elm cells and that its cytotoxic effect is mediated by binding (Fig. 1).

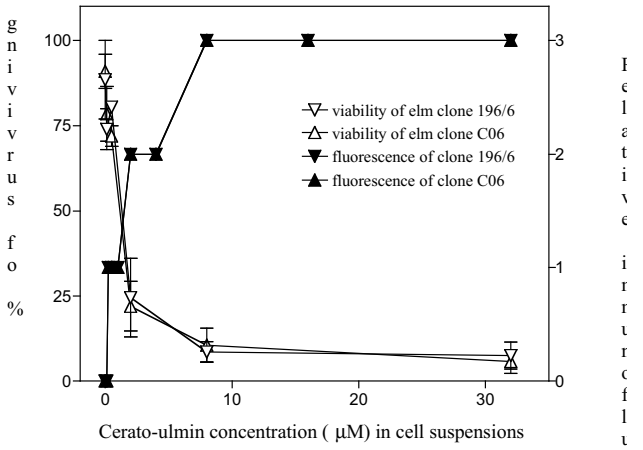

Fig. 1. Effect of cerato-ulmin on cell viability (open triangles) and relative fluorescence index (solid triangles) of immunostained elm cells of a Dutch elm disease susceptible (C06) and a moderately resistant (196/6) elm clone as a function of increasing cerato-ulmin concentration in 10 ml of a $5 \cdot 10^4$ ml^{-1} cell suspension after a three day incubation. The anti-cerato-ulmin antibodies were visualized by incubating with a fluorescein-conjugated antirabbit IgG antiserum and viewed under a fluorescence microscope. Data on cell survival are the mean ± SE of values obtained from samples collected from three replicates obtained in two independent experiments. IF data are based on observation of cells present in 30 microscopy fields in three independent experiments.

The amount of bound CU after 3 days of incubation in the presence of elm cells was determined by semi-quantitative ELISA in cell extracts. Fig. 2 reports the amount of CU bound to elm clones C06 and 196/6 cells, as a function of the initial concentration of CU added to the culture medium. The equation of the saturation binding curve indicated that the maximum quantity of bound CU was 351.92±16.56 (R_2=0.9924) pmoles mg^{-1} d. wt for the clone C06 and 355.59±23.82 (R_2=0.9860) for the clone 196/6. The equilibrium dissociation constant was 7.09±0.89 µM CU for the clone C06 and 8.08±1.39 µM CU for the clone 196/6. The Scatchard plot of the saturation binding curve data (not shown), demonstrated that a linear regression was obtained with the same transformed data.

Both fennel and pear cells did not display fluorescence after a 6 day incubation with purified CU and CU concentration in culture fluids did not significantly decrease over the whole period of incubation.

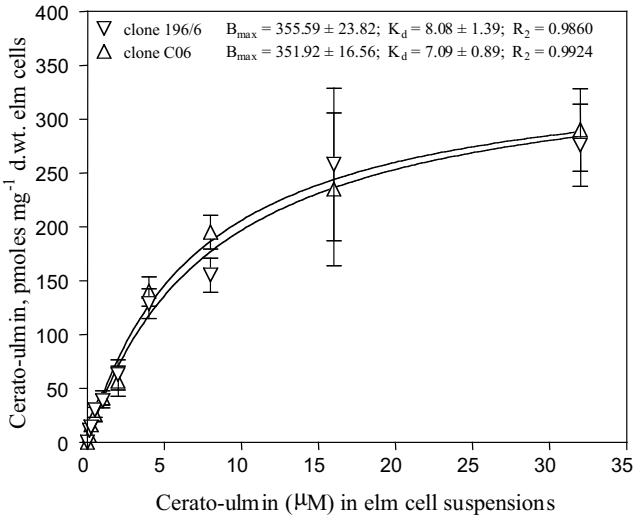

Fig. 2. Cerato-ulmin binding to cells of elm clones C06 and 196/6 cells as a function of increasing cerato-ulmin concentration in 10 ml of a $5{\cdot}10^4$ ml^{-1} cell suspension after a three day incubation. Data regarding cerato-ulmin binding are the mean ± SE of values obtained from samples collected from three replicates obtained in two independent ELISA determinations.

Elm cells support growth of *Ophiostoma* fungi and CU production in dual cultures. Table 1 shows growth and CU production by *O. novo-ulmi* ssp. *americana* isolate H328 and *O. ulmi* isolate E2 in *Ophiostoma*-elm cell dual cultures, prepared utilizing two different liquid media: a medium routinely used for cultivation of *Ophiostoma* (Takai, 1978; and abbreviatied as TK in Table 1) and a medium routinely used for plant cell cultures (Murashige & Skoog, abbreviated as MS). Previous work (Scala, 1997) showed that MS only allows limited growth of *Ophiostoma* fungi. Addition of yeast extract (=YE) determines a quite abundant growth of *Ophiostoma* fungi and CU production. In the absence of YE in TK medium (TK_0) very poor fungal growth occurs and that at least 0.3 g l^{-1} of YE are necessary to support both fungal growth and CU production (Scala, 1997).

In TK_0, the addition of elm cells of either a DED susceptible (C06) and a moderately resistant (196/6) clone to TK_0 supported growth of *Ophiostoma* fungi and CU production, thus fully replacing the effect of YE. In MS the presence of elm cells in MS did not cause a significant effect on fungal growth. The density of blastoconidia reached by both fungi was, in nearly all cases, significantly lower in MS-based than in TK-based media.

CU production was strongly influenced by the culture media. *O. ulmi* did not produce detectable amounts of CU in TK_0. The addition of C06 or 196/6 elm cells or fennel cells, but not pear cells to TK_0 or MS determined production of CU to levels similar to those observed in TK or in TK0.3YE. *O. novo-ulmi* produced no or very low amounts of CU in TK_0 and MS, whereas in TK0.3YE and in MS2YE *O. novo-ulmi* produced relatively large quantities of CU, not significantly different from those produced in the standard medium TK. The addition of elm cells of both clones or fennel cells, but not pear cells to TK_0 and MS caused marked increases in CU production by *O. novo-ulmi*, even if differences were not statistically significant for MS-based media. IF tests performed on plant cells after 6 days of co-culture (Table 1) indicated that CU produced in dual cultures

Table 1. Cell density and cerato-ulmin concentration, as assayed by the turbidimetric method and expressed as cerato-ulmin production index, of *O. novo-ulmi* H328 and *O. ulmi* E2 after 6 days of growth in different Takai- or Murashige & Skoog-based media. Relative IF index (on the basis of a 0-3 arbitrary scale, see Materials and Methods) of cells determined on elm, fennel or pear cells are also indicated. Data are the mean±SE of values obtained from samples collected from three replicates obtained in two independent experiments. Values marked with different small letters are significantly different within TK-based media. Values marked with different capital letters are significantly different from values obtained in the TK medium (at $P \leq 0.05$, according to the Tukey test).

Medium and cultured cells	*Ophiostoma novo-ulmi H328*			*Ophiostoma ulmi E2*		
	Cell Density	Cerato-ulmin production	IF Index	Cell Density	Cerato-ulmin production	IF index
TK	61±11 aA	354±61 aA	-	60±8 aA	9±2 aA	-
TK_0	0.1±0.1 b	0 b	-	0.3±0.1 b	0 b	-
TK_0 + 0.3 g l^{-1}YE	90±9 a	315±41 a	-	90±6 a	8±2 a	-
TK_0 + 196/6 elm cells	100±9 a	270±54 a	3	80±11 a	7±1 a	1
TK_0 + C06 elm cells	94±4 a	287±55 a	3	100±9 a	8±2.5 a	1
TK_0 + fennel cells	75±15 a	189±18 a	0	80±12 a	7±0.5 a	0
TK_0 + pear cells	81±10 a	0 b	0	74±16 a	0 b	0
MS	2±0.3 B	2±1 B	-	2±0.5 C	0 B	-
MS + 2 g l^{-1}YE	45±5 A	299±68 A	-	20±2 B	6±1 A	-
MS + 196/6 elm cells	10±1 B	44±2 B	2	5±1 C	6±0.5 A	1
MS + C06 elm cells	10±1 B	354 B	2	5±1 C	5±0.5 A	1
MS + fennel cells	9±4 B	41±4 B	0	4±2 C	5±1 A	0
MS + pear cells	10±7 B	0 B	0	5±1 C	0 B	0

Table 2. Comparison of the amounts of cerato-ulmin (as assayed by the turbidimetric method and expressed as cerato-ulmin production index) produced in three different TK-based media (see Materials and Methods) by several isolates of *O. novo ulmi* and *O. ulmi* after 6 days of culture. Data are the mean±SE of values obtained from samples collected from three replicates obtained in two independent experiments. Within each line, different letters indicate that values are significantly different at $P \leq 0.05$, according to the Tukey test.

Fungal isolates	Cerato-ulmin production		
	TK	TK_0	TK_0 + elm cells
O. novo ulmi			
ssp. *novo-ulmi*			
H 328	351±19 a	0 b	271±85 a
H 327	241±17 a	0 b	185±23 a
ssp. *americana*			
182	220±19 a	0 b	192±73 a
H 162	221±16 a	0 b	154±44 a
H 351	247±46 a	0 b	156±18 a
O. ulmi			
E 2	8±2 a	0 b	6±1 a
R 21	11±2 a	0 b	7±1 a
Yu 99	6±1 a	0 b	5±1 a
Yu 16	4±1 a	0 b	6±1 a

bound to elm but not to fennel or pear cells, giving a signal proportional to the amount of CU present in the culture medium.

The ability of elm cells to support both fungal growth and CU production was confirmed on a set of isolates representative of *O. novo-ulmi* ssp. *novo-ulmi* and *americana* and *O. ulmi* (Table 2). The positive effect of elm and fennel but not pear cells to support CU production indicates that elm and fennel but not pear cells apparently have an inducer of CU production.

DISCUSSION

In this paper we report the occurrence of binding and cytotoxicity of CU to elm cells. CU binding to elm but not to fennel and pear cells, two non-hosts to DED pathogens, strongly supports the assumption that CU is a pathogenicity factor for DED fungi. The binding curve obtained with increasing CU concentrations vs. a standard elm cell suspension shows that cells are saturated with about 360 pmoles CU mg^{-1} d. wt elm cells and the K_d determined for the binding of CU to elm cells ranges between 7.09 and 8.08 µM for a susceptible and a moderately resistant elm clone. The occurrence of similar values in the binding parameters for the two elm clones tested does not indicate, at least in our system, that CU is a virulence factor in DED. The values, however, could be overestimated since in aqueous solution CU molecules can aggregate to each other (Russo *et al.*, 1982). This can explain, at least in part, why the K_d determined for CU binding to elm cells is much higher than the K_d determined for some other plant receptor-ligand combinations (*e.g.* Kooman-Gershmann *et al.*, 1996). More investigation is needed to establish if CU simply adsorbs to elm cell surface or if specific receptor molecules are involved. Work is in progress to study the influence of culture conditions on CU binding in terms of temperature, ionic strenght and pH. In the present work we could also observe that the binding was not affected by several washes in 0.15M NaCl and distilled water. The intensity of fluorescence of elm cells and their mortality were correlated to the amount of CU added to the incubation mixture. On the basis of our results and previous observation that leaf wilt correlated to the amount of CU in leaf tissues (Scala *et al.*, 1997b), it can be proposed that CU is produced by the DED fungi in xylematic vessels and is then translocated in leaves where it binds to host cells. The binding would be the preliminary step in causing cellular damage and consequent leaf wilting. Mutual molecular interactions between cell surfaces of host plants and pathogens, and associated signalling mechanisms, are considered to play a fundamental role in pathogenesis (Callow *et al.*, 1988). Presence of receptors at the surface of plant cells for fungal signal molecules was proposed as a logical presumption (Ride, 1992), since cell surface receptors are a common theme in cell-cell recognition events (Chapman *et al.*, 1988). Ride (1992) reviewed various plant-pathogen systems, where some indirect or direct evidence for plant cell receptors have been obtained. Binding of elicitor molecules to plasma membrane receptors as a preliminary step in recognition events leading to hypersensitive response has been described for a peptide elicitor of *Phytophtora megasperma* (Nurnberger *et al.*, 1994) and the race-specific AVR9 elicitor of *Cladospori-*

um fulvum (Kooman-Gershmann *et al*., 1996). The characterization of specific receptors for fusicoccin, a phytotoxin produced by the fungus *Fusicoccum amygdali*, a pathogen of almond, peach and related species, and their role in mediating physiological response(s) of host cells to the ligand, has been of a great interest for both plant physiology and plant pathology (Graniti *et al*., 1995). Protein receptor for victorin, a host selective toxin produced by the oat pathogen *Cochliobolus victoriae* has also been extensively characterised (Wolpert *et al*., 1994). In both cases, the study of toxin receptors contributed to a better understanding to the mechanism of action of the toxin and/or the physiological response(s) to the ligand. The occurrence and significance of elm cell receptor(s) for CU in the pathogenic process of DED and in disease symptoms expression, needs more evidence to be elucidated.

On the basis of our results we cannot, at present, exclude that elm cells could lack a specific receptor for CU and that cellular damage could simply derive from the deposition and adhesion of one or more layers of CU on the external surface of elm cell walls, hampering cellular permeability. Experiments with structural analogues of CU should give more information about the type of interaction between CU and elm cells. It also has to be mentioned that CU shares with β-amyloid, a protein causing a number of neurological and degenerative diseases by deposition on cells, the ability to self-assembly and to determine the formation of aggregates (Lorenzo and Yankner, 1994).

Our data confirm Takai's results (1978) that cultural conditions influence CU production. The addition of yeast extract was essential for CU production in both TK_0 e MS. We found that elm cells fully replaced yeast extract in TK_0, by supporting both abundant fungal growth and CU production. In the presence of elm cells, *O. novo-ulmi* produced quantities of CU always higher than those of *O. ulmi*. This result was obtained by testing several isolates belonging to both subspecies of *O. novo-ulmi* and to *O. ulmi*, reflecting the behaviour of the two species observed in Takai-based media (Brasier, 1991; Scala *et al.*, 1997a) and *in planta* (Scala *et al.*, 1997b). In MS the effect of elm cells on CU production was much less pronounced than in Takai-based media, but still evident. It should be considered that MS is an unusual medium for growing fungi, since it has been formulated for *in vitro* growth of plant callus and cell cultures.

In an attempt to establish a pathogenicity test alternative to whole plant inoculation, Scala *et al.* (1996) found no differences between the degree of colonization of either DED pathogens of calli derived from a susceptible (*U. carpinifolia*) and a resistant (*U. pumila*) elm species. Nonetheless, calli of the susceptible species were more subjected to cell plasmolysis than those of the resistant species. In our experimental system, which also considers elm clones having different levels of susceptibility to DED pathogens, no differences were found in sensitivity to CU. Presumably, during growth on calli, factors other than CU could be responsible for the differential sensitivity to plasmolysis observed by Scala *et al*. (1996).

Both fennel and pear cells were able to support fungal growth, but only fennel cells induced CU production, indicating that, in the absence of a source of organic nutrients like yeast extract, CU production is not constitutive and does not depend on a elm-specific inducer molecule. More investigation is needed to establish if pear cell culture media lack one or more metabolites necessary for CU production or contain specific suppressors.

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